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Time-Dependent Activation of Phox2a by the Cyclic AMP Pathway Modulates Onset and Duration of p27^{Kip1} Transcription▽†

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In noradrenergic progenitors, Phox2a mediates cell cycle exit and neuronal differentiation by inducing p27^{Kip1} transcription in response to activation of the cyclic AMP (cAMP) pathway. The mechanism of cAMP-mediated activation of Phox2a is unknown. We identified a cluster of phosphoserine-proline sites in Phox2a by mass spectrometry. Ser206 appeared to be the most prominent phosphorylation site. A phospho-Ser206 Phox2a antibody detected dephosphorylation of Phox2a that was dependent on activation of the cAMP pathway, which occurred prior to neuronal differentiation of noradrenergic CAD cells. Employing serine-to-alanine and serine-to-aspartic acid Phox2a substitution mutants expressed in inducible CAD cell lines, we demonstrated that the transcriptional activity of Phox2a is regulated by two sequential cAMP-dependent events: first, cAMP signaling promotes dephosphorylation of Phox2a in at least one site, Ser206, thereby allowing Phox2a to bind DNA and initiate p27^{Kip1} transcription; second, following dephosphorylation of the phosphoserine cluster (Ser202 and Ser208), Phox2a becomes phosphorylated by protein kinase A (PKA) on Ser153, which prevents association of Phox2a with DNA and terminates p27^{Kip1} transcription. This represents a novel mechanism by which the same stimulus, cAMP signaling, first activates Phox2a by dephosphorylation of Ser206 and then, after a built-in delay, inactivates Phox2a via PKA-dependent phosphorylation of Ser153, thereby modulating onset and duration of p27^{Kip1} transcription.

Formation of the proper structures of the nervous system involves coordination of cell cycle exit of neural progenitors with differentiation (9). For example, the development of the organ of Corti of the inner ear (23) and neurogenesis in the spinal cord (19) involve transcription of the cyclin-dependent kinase inhibitor p27^{Kip1}, which coordinates cell cycle exit of neural progenitors with differentiation. In the catecholaminergic CAD cell line, the transcription of p27^{Kip1} is mediated by the homeodomain (HD) transcription factor Phox2a (29), and in neural spinal cord progenitors it is mediated by the HD transcription factor Cux2 (19). The mechanism by which the cell cycle apparatus is coordinated with the transcription of p27^{Kip1} has not yet been determined. To understand this mechanism, it is important to decipher how lineage-determining transcription factors such as Phox2a or Cux2 become activated by extracellular inductive signals. A prevalent mechanism modulating activation of transcription factors in response to extracellular signals is reversible phosphorylation/dephosphorylation (11, 17), which regulates DNA binding (21), transactivation (12), stability, or nuclear localization (31). In addition, transcription factors undergo multisite phosphorylations that fine-tune their activity to reflect the strength of the inducing signal (8, 30). In this study, we investigated the mechanism by which inductive signals modulate the activity of Phox2a to initiate transcription of p27^{Kip1}. An inductive signal for noradrenergic neuron differentiation in vitro is cyclic AMP (cAMP) signaling (4, 7), which regulates Phox2a activation (29) by a mechanism not yet determined.

Phox2a is required for differentiation of noradrenergic neurons in the central nervous system (CNS) and peripheral nervous system (PNS) (25, 28, 35). Noradrenergic neurons express the catecholamine biosynthetic enzymes tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (2, 13). Phox2a directly regulates the transcription of both the TH and dopamine-β-hydroxylase genes (42) in response to cAMP signaling (1, 7, 37). The major noradrenergic center in the CNS is the locus ceruleus, which is absent in Phox2a$^{-/-}$ mice (28) and MASH1$^{-/-}$ mice (16). MASH1, induced by BMP2 signaling, is a proneural transcription factor (32, 33) required for Phox2a expression (16). Noradrenergic neurons of the PNS include sympathetic ganglia and parenchymal cells of the adrenal medulla. Adrenal glands of mice lacking MASH1 exhibit reduced numbers of Phox2a-positive and TH-expressing cells despite expression of the paired HD transcription factor Phox2b (16).

Although the developmental origins of noradrenergic progenitors of the CNS (ventricular zone) and PNS (trunk-derived neural crest cells) are distinct (18, 22), in vivo (16) and in vitro (3, 7, 24, 29) studies indicate that the mechanism of noradrenergic differentiation mediated by Phox2a is the same. In vitro models of CNS- and PNS-derived noradrenergic differentiation include primary cultures of neural crest cells and cultured CAD cells (3, 5, 7, 29). The CAD cell line is a variant of the

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Cath.a cell line derived from locus ceruleus brain tumors of transgenic mice expressing the simian virus 40 antigen from the TH promoter (36). Using these cellular models, it has been demonstrated that, in addition to BMP2, moderate activation of the cAMP pathway is required for both transcription and activation of Phox2a (3, 7, 29). Activated Phox2a mediates neuronal differentiation of primary neural crest cells and CAD cells (4, 5, 24).

cAMP signaling regulates the binding of Phox2a to DNA (1, 29, 37) and the ability of Phox2a to mediate transcription of the $p27^{Kip1}$ gene (29). Treatment with the phosphatase inhibitor okadaic acid (OA) inhibited cAMP-induced binding of Phox2a to the $p27^{Kip1}$ promoter, the transcription of $p27^{Kip1}$ mRNA, and neuronal differentiation of neural crest and CAD cells (7, 29).

In this study, we employed the CAD cell line to investigate the mechanism by which cAMP signaling regulates the activity of Phox2a to induce $p27^{Kip1}$ transcription. We determined by mass spectrometry (MS) that Ser206, located within a cluster of proline-directed phosphoserines (Ser202 and Ser208), is the most prominent phosphorylation site of Phox2a. We provide evidence that cAMP signaling promotes the dephosphorylation of Ser206, which allows Phox2a to bind to the $p27^{Kip1}$ promoter and mediate $p27^{Kip1}$ transcription. In primary neural crest cells and CAD cells, transcription of $p27^{Kip1}$ in response to activation of the cAMP pathway occurs in a defined interval prior to neuronal differentiation (29). We demonstrate that protein kinase A (PKA) phosphorylates Ser153 located adjacent to the Phox2a HD and that this phosphorylation determines the interval of $p27^{Kip1}$ transcription. PKA-mediated phosphorylation of Ser153 occurs after the dephosphorylation of the phosphoserine cluster (Ser202, Ser206, and Ser208) and interferes with the binding of Phox2a to the $p27^{Kip1}$ promoter, thereby terminating $p27^{Kip1}$ transcription. Thus, cAMP signaling first activates Phox2a by dephosphorylation of Ser206 and then, following dephosphorylation of Ser202 and Ser208, inactivates Phox2a by PKA-mediated phosphorylation of Ser153, thereby modulating both onset and duration of $p27^{Kip1}$ transcription.

MATERIALS AND METHODS

Cell culture. Growth conditions for CAD cells were as described previously (5). Neuronal differentiation of CAD cells in serum-containing medium was induced by addition of 5 μM forskolin (5). $p27^{Kip1}$ small interfering RNA (siRNA) transfections and controls were performed as described previously (29). Tetracycline-regulated CAD cell lines expressing wild-type (WT) Phox2a-FLAG or site-directed Phox2a-FLAG mutants were constructed as described previously (29). Phox2a mutants containing serine (S)-to-alanine (A) or serine-to-aspartic acid (D) substitutions were generated using the QuikChange XL site-directed mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing.

Phox2a-FLAG purification and MS. Phox2a-FLAG was expressed in the tetracycline-regulated CAD-Phox2a-FLAG cell line (29) by tetracycline removal and purified by immunoaffinity chromatography using anti-FLAG M2 affinity gel (Sigma) and elution with FLAG peptide (Sigma). Phox2a-FLAG preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Peptides were generated by in-gel digestion with trypsin and analyzed by liquid chromatography-tandem MS using an Agilent 1100 capillary high-pressure liquid chromatograph and either an LTQ ion trap (Thermo Scientific) or a 4800 matrix-assisted laser desorption ionization–time-of-flight MS exactly as described previously (14). Sorcerer (Sage-N Research) or Mascot (Matrix Science) database search software was used to identify potential Phox2a phosphopeptides. All spectra were analyzed manually to confirm peptide identity and localize phosphate groups.

Assays. Real-time PCR quantification of $p27^{Kip1}$ mRNA was performed as described previously (29). Chromatin immunoprecipitation (ChIP) assays employed primer pairs corresponding to HD site 3 of the $p27^{Kip1}$ promoter (29). Southwestern blot analysis was performed as described by Handen and Rosenberg (15) with minor modifications. Immunofluorescence microscopy and immunoblot analyses were performed as described previously (29).

Antibody generation. Phospho-S206 Phox2a antibody was generated by Proteintech Group Inc. using the synthetic peptide SLASPRLpSPSPLPAA as the immunizing antigen. The phospho-S206 Phox2a antibody was used at dilutions of 1:500 for immunofluorescence microscopy and 1:1,000 for immunoblotting.

In vitro PKA phosphorylation. Purified WT Phox2a-FLAG and S153APhox2a-FLAG proteins were incubated in 50 mM HEPES (pH 7.4), 5 mM $MnCl_2$, and 100 mM ATP with 10 U PKA catalytic subunit (Sigma) and 20 μCi [γ-^{32}P]ATP (6,000 Ci/mmol) at 30°C for 30 min. Phosphoproteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by autoradiography. For alkaline gel electrophoresis, the region of the nitrocellulose membrane containing the phosphorylated protein was excised and incubated in 0.5% polyvinylpyrrolidone (PVP-360; Sigma) and 100 mM acetic acid for 30 min at 37°C. After five washes in H_2O, membranes were incubated for 2 h at 37°C with 10 μg L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) in 50 mM NH_4HCO_3. Fresh trypsin (10 μg) was added and incubated for an additional 2 h. Samples were lyophilized and resuspended in alkaline PAGE sample buffer (0.125 M Tris-HCl [pH 6.8], 6 M urea, and bromophenol blue). Tryptic phosphopeptides were separated by alkaline 40% PAGE at 8 mA (10, 20); phosphopeptides were detected by autoradiography.

Metabolic labeling. A total of 2×10^6 CAD cells expressing S202A/S206A/S208APhox2a-FLAG, S206APhox2a-FLAG, or S153A/S206APhox2a-FLAG for 72 h were preincubated in phosphate-free Dulbecco modified Eagle medium for 1 h, followed by incubation in medium containing 0.6 mCi/ml [^{32}P]orthophosphate with or without addition of forskolin (5 μM) and H89 (10 μM). Cells were lysed in radioimmunoprecipitation assay buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.2), 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 100 mM NaF, 50 mM β-glycerolphosphate, and protease inhibitor cocktail (1:1,000; Sigma).

RESULTS

Identification of Phox2a phosphorylation sites by MS. To understand the mechanism of Phox2a activation, we investigated the modifications of Phox2a by MS. We employed purified Phox2a-FLAG protein isolated from a CAD cell line that expresses ectopic Phox2a-FLAG via the tetracycline-regulated (Tet-off) expression system (29). Phox2a-FLAG-expressing CAD cells were grown without activation of the cAMP pathway.

To identify Phox2a phosphorylation sites, tryptic peptides of purified Phox2a-FLAG were analyzed by high-pressure liquid chromatography and tandem MS on a linear ion trap MS. We obtained high-quality fragment ion spectra covering nearly 90% of the Phox2a sequence. A cluster of proline-directed phosphorylation sites was detected in the C-terminal region. Two individual peptides, $Phox2a^{179–204}$ and $Phox2a^{205–224}$, were each detected in singly and doubly phosphorylated forms (Fig. 1; see Fig. S1 in the supplemental material). Fragment ion spectra allowed conclusive localization of phosphate groups to Ser202, Ser206, and Ser208 within these two peptides (Fig. 1). An additional site was localized to either Ser183 or Thr185. The latter site is minor compared to Ser202, because the singly phosphorylated $Phox2a^{179–204}$ peptide was exclusively modified at Ser202 (see Fig. S1B in the supplemental material). On the other hand, the singly phosphorylated $Phox2a^{205–224}$ peptide appeared to be a mixture of species phosphorylated on Ser206 and Ser208 (see Fig. S1A in the supplemental material).

Two qualitative observations suggested that Ser206 was the most prominent phosphorylation site. First, when the same

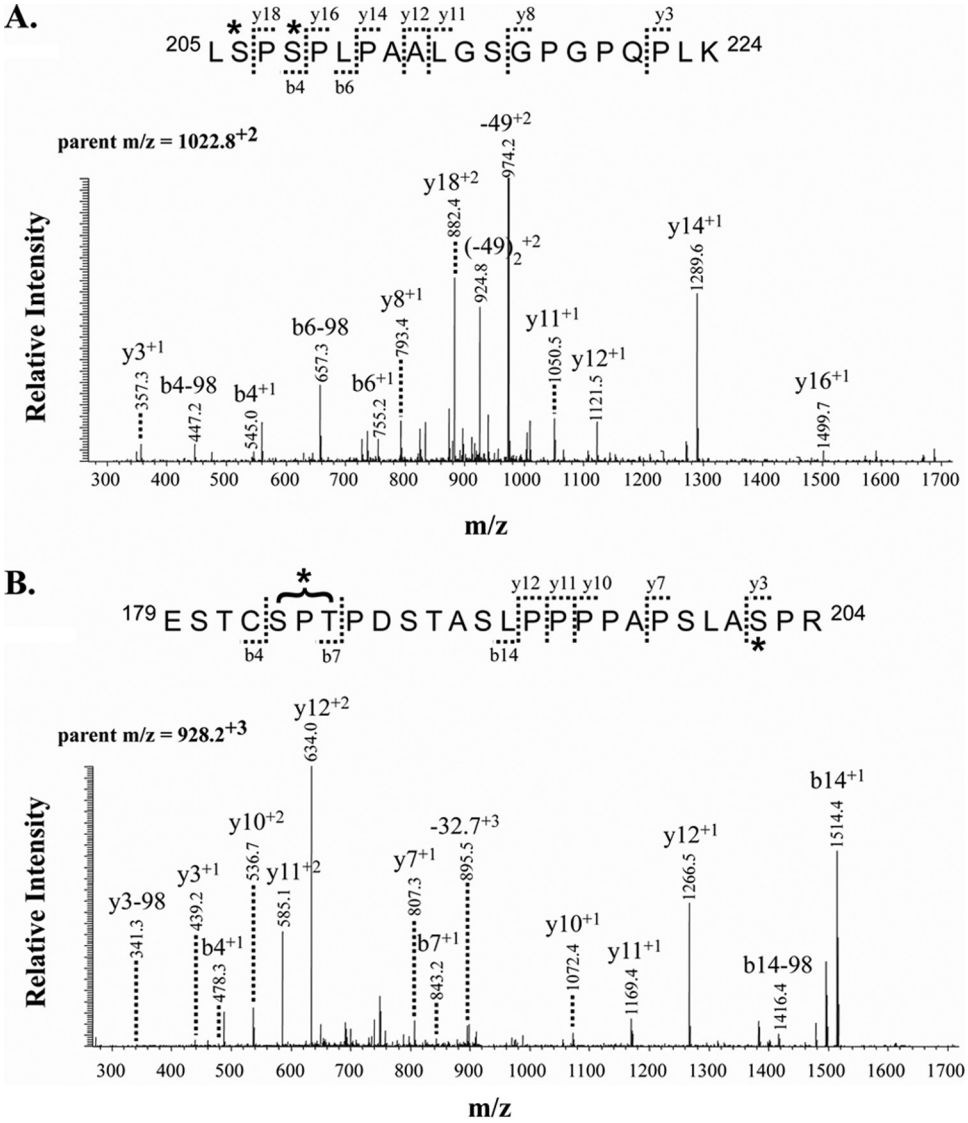

FIG. 1. Phox2a is phosphorylated in vivo at a cluster of S-P sites. Tandem MS was used to map in vivo phosphorylation sites on affinity-purified FLAG-Phox2a. Only a subset of y and b ions is labeled. (A) Doubly charged, doubly phosphorylated Phox2a$^{205–224}$ tryptic peptide. The y ion series up to y16 is unmodified, suggesting that Ser206 and Ser208 are the two sites of phosphorylation. This is confirmed by the b4 and b6 ions, which are shifted by *m*/*z* of 160 and associated with peaks representing neutral loss of phosphoric acid (−98). (B) Triply charged, doubly phosphorylated Phox2a$^{179–204}$ tryptic peptide. The y3 ion is shifted by *m*/*z* of 80 and is associated with a peak representing neutral loss of 98, demonstrating that Ser202 is phosphorylated. The b14 and b7 ions are also shifted by *m*/*z* of 80, but b4 is unmodified, localizing a second phosphorylation site to Ser183 and/or Thr185. In both panels, the peptide sequence highlights the fragment ions labeled in the spectra and asterisks mark the locations of phosphate groups. See Fig. S1 in the supplemental material for comparison of unmodified and phosphorylated peptide spectra.

Phox2a tryptic peptides were analyzed by matrix-assisted laser desorption ionization tandem MS, the fragment ion spectrum for phospho-Phox2a$^{205–224}$ revealed no evidence of phosphorylation at Ser208 (see Fig. S1C in the supplemental material). Second, based on extracted ion chromatograms (not shown), the ratio of phosphopeptide signal to unmodified peptide signal for Phox2a$^{205–224}$ was much greater than that for Phox2a$^{179–204}$. This result is consistent with the stoichiometry of Phox2a$^{205–224}$ phosphorylation being significantly greater than that of Phox2a$^{179–204}$. These observations suggest that Ser206 is the most prominent site of phosphorylation. Accordingly, we focused our initial efforts on studying the phosphorylation of Ser206.

Phox2a Ser206 is dephosphorylated in response to cAMP signaling. We generated a phospho-S206-specific Phox2a polyclonal antibody (see Materials and Methods). In immunoblots the phospho-S206 Phox2a antibody detected purified Phox2a-FLAG protein and Phox2a-FLAG in lysates of cells grown without treatment with forskolin (Fig. 2A). In contrast, immunodetection was abolished after treatment of purified Phox2a-FLAG protein with λ phosphatase, by addition of the competing phospho-S206 Phox2a peptide, or using a Phox2a mutant

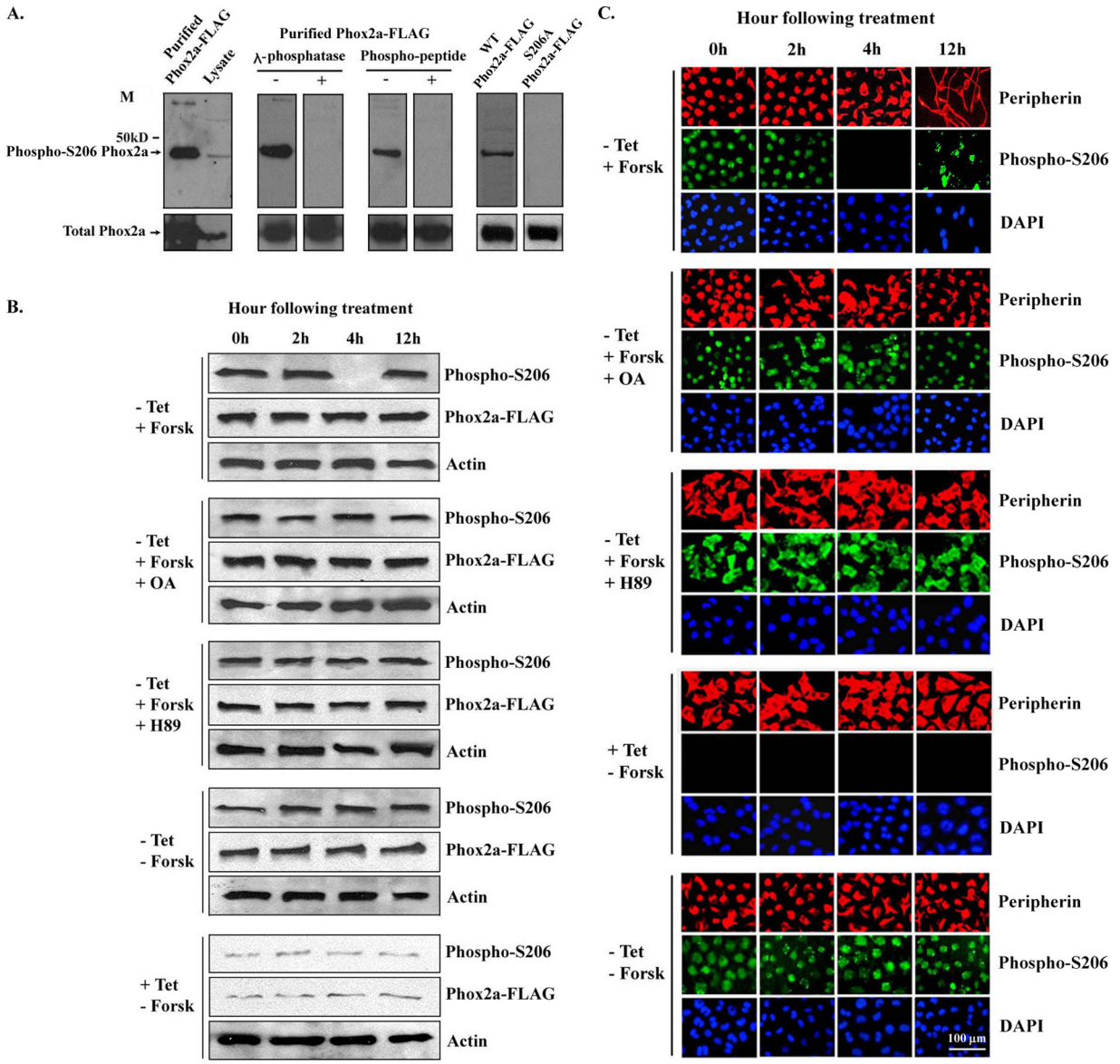

FIG. 2. (A) Immunoblots using a phospho-S206 Phox2a antibody (see Materials and Methods) and purified Phox2a-FLAG protein or lysate (20 μg) from Phox2a-FLAG-expressing CAD cells, purified Phox2a-FLAG treated without (−) and with (+) λ phosphatase or with phospho-S206 Phox2a peptide, and FLAG immunoprecipitates of WT Phox2a-FLAG and S206APhox2a-FLAG. M, molecular mass. (B) Immunoblots using the phospho-S206 Phox2a antibody of lysates from CAD-Phox2a-FLAG cells grown with (+) or without (−) tetracycline for 72 h and treated for an additional 0 to 12 h with (+) or without (−) forskolin (5 μM), OA (5 nM), or H89 (10 μM), as indicated. Total Phox2a-FLAG was detected by FLAG immunoblotting. An actin immunoblot was used as the loading control. (C) Immunofluorescence microscopy of CAD-Phox2a-FLAG cells grown with (+) or without (−) tetracycline for 72 h and treated for an additional 0 to 12 h with (+) or without (−) forskolin (5 μM), OA (5 nM), or H89 (10 μM), as indicated; cells were coimmunostained with phospho-S206 Phox2a antibody (green) and peripherin antibody (red). Merged images also show staining of DNA with 4′,6′-diamidino-2-phenylindole (DAPI).

protein with alanine substitution of Ser206 (Fig. 2A). These results demonstrate the specificity of the phospho-S206 antibody for Phox2a phosphorylated on Ser206.

The tetracycline-regulated CAD-Phox2a-FLAG cell line exhibits peak expression of $p27^{Kip1}$ mRNA at 4 h after forskolin stimulation (29). To investigate whether Ser206 phosphorylation is linked to the transcriptional activity of Phox2a in this cell line, we examined the phosphorylation status of Phox2a on Ser206 by immunoblots with the phospho-S206 antibody (Fig. 2B). In a time course after treatment with forskolin, Ser206 phosphorylation was detected at 2 h, whereas detection was abolished by 4 h. Interestingly, Ser206 was found to be phosphorylated at 12 h after treatment with forskolin. Cotreatment with forskolin and the phosphatase inhibitor OA (5 nM) or the PKA inhibitor H89 (10 μM) restored Ser206 phosphorylation at 4 h. Moreover, Ser206 is phosphorylated without activation of the cAMP pathway (Fig. 2B).

The CAD-Phox2a-FLAG cell line undergoes neuronal differentiation at 12 h following treatment with forskolin, as detected by peripherin immunostaining of neurites; treatment

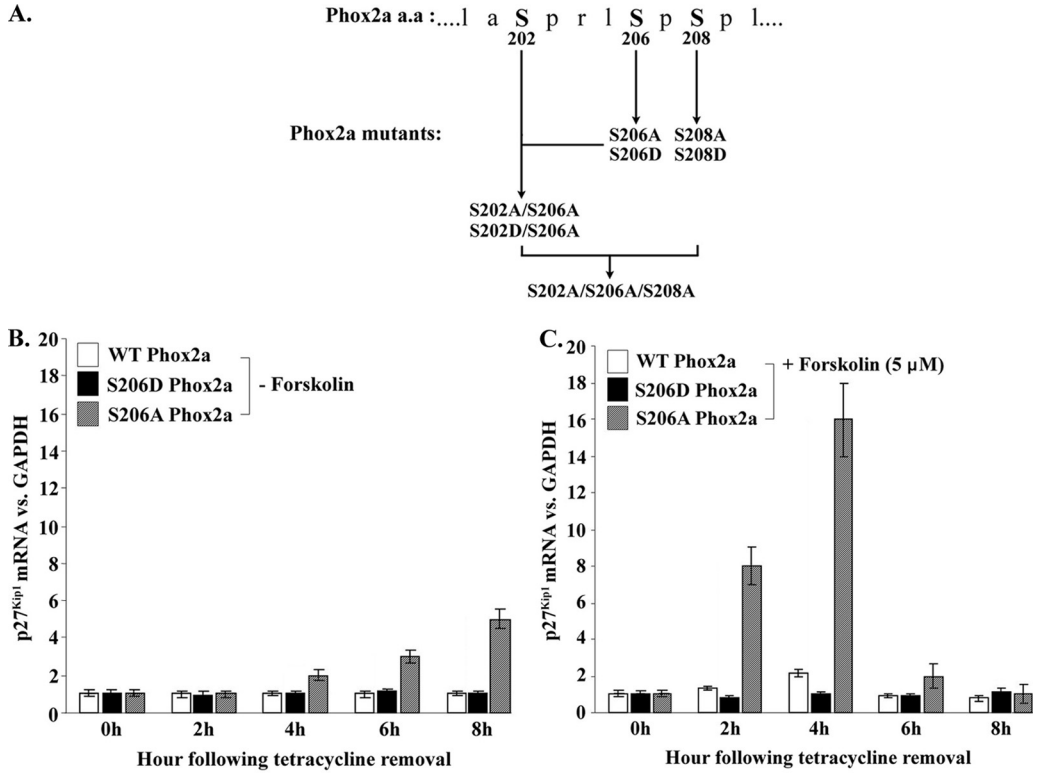

FIG. 3. (A) Site-directed Phox2a mutants expressed in stable CAD cell lines via the Tet-off system (see Fig. S2 in the supplemental material). (B and C) Real-time PCR quantification of endogenous $p27^{Kip1}$ mRNA using total RNA isolated from the indicated CAD-Phox2a-FLAG cell lines. The indicated Phox2a proteins were expressed in a time course (0 to 8 h) by tetracycline removal and concurrent treatment without (−) forskolin (B) and with (+) forskolin (5 μM) (C). Phox2a protein levels are shown in Fig. S2 in the supplemental material. Three independent RNA isolations were analyzed by PCR using identical triplicates. Quantification used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Fold induction is relative to 0 h. Error bars indicate standard deviations.

with OA or H89 inhibits cAMP-induced neuronal differentiation (7, 29). To investigate the phosphorylation status of Ser206 relative to neuronal differentiation, Phox2a-FLAG-expressing CAD cells treated with forskolin for 12 h were analyzed by immunofluorescence microscopy, using antibodies for phospho-S206 Phox2a and peripherin (Fig. 2C). Ser206 is phosphorylated at 2 h, but not at 4 h, following addition of forskolin. Interestingly, upon neuronal differentiation occurring at 12 h after treatment, Ser206 is phosphorylated. Cotreatment with forskolin and either OA or H89 maintained Ser206 phosphorylation at 4 h, in agreement with the immunoblot data (Fig. 2B), and inhibited neuronal differentiation. These results suggest that dephosphorylation of Ser206 mediated by the cAMP pathway is necessary for neuronal differentiation of CAD cells.

Dephosphorylation of Ser206 activates Phox2a-mediated $p27^{Kip1}$ transcription. To directly test the functional significance of phospho-Ser206 for Phox2a activity, we generated Phox2a mutants with serine-to-alanine or serine-to-aspartic acid substitutions at that site. Since Ser202 and Ser208 are also phosphorylated (Fig. 1), we examined combinations of the Ser206 mutations with alanine or aspartic acid substitutions of Ser202 and Ser208 as well (Fig. 3A). We then constructed tetracycline-regulated CAD cell lines expressing each of the Phox2a-FLAG mutants. These CAD cell lines were used for all subsequent experiments. The introduced mutations do not affect the expression level of the mutant Phox2a proteins (see Fig. S2 in the supplemental material). The earliest known transcriptional target of Phox2a is the $p27^{Kip1}$ gene (29). Accordingly, we investigated the activity of the Phox2a mutants by quantifying expression of endogenous $p27^{Kip1}$ mRNA as a function of time by real-time PCR, with and without treatment with forskolin.

First, we studied the activity of S206DPhox2a and S206APhox2a mutants (Fig. 3B and C). Without activation of the cAMP pathway, neither WT nor S206DPhox2a induced $p27^{Kip1}$ mRNA. However, S206APhox2a mediated a progressive increase in $p27^{Kip1}$ mRNA from 2 to 8 h (Fig. 3B), suggesting that dephosphorylation of Ser206, modeled by the alanine substitution, renders Phox2a constitutively active. Conversely, Ser206DPhox2a failed to induce $p27^{Kip1}$ mRNA expression even with activation of the cAMP pathway (Fig. 3C). Since aspartic acid can often mimic phosphoserine, these results suggest that Ser206 phosphorylation inhibits Phox2a-mediated transcription.

Interestingly, upon activation of the cAMP pathway, $p27^{Kip1}$ expression in Ser206APhox2a cells was no longer constitutive but became time dependent. Specifically, a peak of $p27^{Kip1}$ mRNA (16-fold increase) was observed at 4 h, followed by a decrease to the basal level by 6 h (Fig. 3C). This pattern of $p27^{Kip1}$ mRNA expression resembles that in WT Phox2a-FLAG-expressing cells, but the magnitude is greater. Similar

results were obtained with both S208APhox2a (see Fig. S3A and B in the supplemental material) and S202A/S206APhox2a (see Fig. S4A and B in the supplemental material) after treatment with forskolin. These results strongly suggest that cAMP signaling mediates an additional Phox2a regulatory mechanism that determines the duration of Phox2a activation.

cAMP signaling terminates Phox2a activation via Ser153 phosphorylation. To investigate how cAMP signaling terminates Phox2a activation, we focused on Ser153, a potential phosphorylation site for PKA located adjacent to the DNA binding domain of Phox2a (38). Unfortunately, peptides containing Ser153 have not been detected in our MS analyses. Nevertheless, we generated S153A and S153D substitutions in WT and S206APhox2a and constructed tetracycline-regulated CAD cell lines expressing these mutants (Fig. 4).

Neither S153APhox2a nor S153DPhox2a induced expression of $p27^{Kip1}$ mRNA without activation of the cAMP pathway (Fig. 4A), in agreement with the requirement for dephosphorylation of Ser206 in Phox2a activation (Fig. 3). In the presence of forskolin, S153APhox2a mediated a progressive increase in $p27^{Kip1}$ mRNA, in contrast to the distinct peak of $p27^{Kip1}$ mRNA induced by WT Phox2a (Fig. 4B). Moreover, S153DPhox2a did not induce expression of $p27^{Kip1}$ mRNA even after activation of the cAMP pathway (Fig. 4B). These results are consistent with a phosphorylation of Ser153 that contributes to the cAMP-dependent termination of Phox2a activity. Immunoblots (Fig. 4C) and immunostaining (Fig. 4D) with the phospho-S206 Phox2a antibody demonstrate that both S153APhox2a and S153DPhox2a proteins become dephosphorylated at Ser206 4 h after forskolin stimulation. Since S153DPhox2a did not induce expression of $p27^{Kip1}$ mRNA even after cAMP-induced dephosphorylation of Ser206 (Fig. 4C), this suggests that Ser153 phosphorylation has a dominant-negative effect on Phox2a activity.

To test the hypothesis that Ser153 phosphorylation has a dominant inhibitory effect on Phox2a activity, we examined the effect of S153A and S153D substitutions in the context of the S206APhox2a mutant. Irrespective of cAMP signaling, S153D/S206APhox2a failed to induce $p27^{Kip1}$ mRNA expression (Fig. 4E and F). In contrast, S153A/S206APhox2a mediated a progressive increase in $p27^{Kip1}$ expression in both the absence (Fig. 4E) and presence (Fig. 4F) of forskolin, reaching nearly a 50-fold induction at 8 h after forskolin treatment. These results strongly suggest that (i) S153 phosphorylation is essential for cAMP-dependent termination of Phox2a activity, as revealed by the S206APhox2a mutant (Fig. 3C), and (ii) Ser153 phosphorylation limits the duration of Phox2a activation.

PKA phosphorylates Phox2a Ser153. To investigate whether Ser153 can be phosphorylated by PKA, we performed in vitro kinase assays employing purified WT Phox2a-FLAG and S153APhox2a-FLAG proteins as substrates (Fig. 5A). WT Phox2a-FLAG was prominently phosphorylated by PKA, whereas minimal phosphorylation was observed with S153APhox2a. To confirm that WT Phox2a-FLAG was phosphorylated on Ser153, the phosphorylated and SDS-PAGE-purified WT and S153APhox2a proteins were isolated and digested with trypsin. The tryptic peptides were analyzed by alkaline 40% PAGE, which permits separation of negatively charged peptides based on the ratio of charge to mass (10, 20). Complete tryptic digestion of Phox2a generates a five-amino-acid peptide containing Ser153 (^{151}AASAK^{155}). Direct comparison of the WT and S153APhox2a tryptic peptide patterns revealed a short ^{32}P-radiolabeled peptide present only in the WT Phox2a protein digest (Fig. 5B).

We examined whether Ser153 is phosphorylated in cells upon activation of the cAMP pathway. CAD cells expressing S202A/S206A/S208APhox2a, S206APhox2a, and S153A/S206APhox2a were metabolically labeled with [^{32}P]orthophosphate as a function of activation of the cAMP pathway. The ^{32}P-radiolabeled Phox2a proteins were purified by immunoaffinity chromatography and SDS-PAGE. Tryptic peptides of ^{32}P-radiolabeled proteins were analyzed by 40% PAGE and autoradiography (Fig. 5C). As with the in vitro PKA phosphorylation pattern of Phox2a, a short ^{32}P-radiolabeled peptide was detected in the tryptic digest of S202A/S206/S208APhox2a isolated from forskolin-stimulated cells. The alanine substitutions in S202A/S206/S208APhox2a minimized ^{32}P incorporation at other phosphorylation sites. The tryptic digest of S206APhox2a which was metabolically labeled with [^{32}P]orthophosphate in the presence of forskolin also generated a prominent, short ^{32}P-radiolabeled peptide. Importantly, cotreatment with the PKA inhibitor H89 abolished detection of this short peptide, indicating that this phosphorylation is mediated by PKA. Significantly, S153A/S206APhox2a, which lacks the potential PKA site at Ser153, also lacks this ^{32}P-radiolabeled peptide, providing evidence that Ser153 is phosphorylated in situ in response to activation of the cAMP pathway.

Dephosphorylation of Ser202, Ser206, and Ser208 modulates the duration of Phox2a activation. To explore the contribution of the other phosphorylation sites identified by MS, the combined effect of Ser202, Ser206, and Ser208 dephosphorylation was tested using the triple-alanine S202A/S206A/S208APhox2a mutant (Fig. 6). This Phox2a mutant exhibited a progressive increase in $p27^{Kip1}$ mRNA from 2 to 8 h in the absence of forskolin, similar to the case for S206APhox2a but 50% greater in magnitude (Fig. 6A). Upon activation of the cAMP pathway, the triple-alanine mutant exhibited the same level of $p27^{Kip1}$ mRNA at the 2-h interval as without activation of the cAMP pathway, but intriguingly, the level of $p27^{Kip1}$ mRNA returned to the basal level by 4 h (Fig. 6B). These observations suggest that with the triple-alanine Phox2a mutant, the cAMP-mediated regulation is inhibition of its transcriptional activity. Interestingly, the double mutant S202A/S206APhox2a also displayed a peak increase in $p27^{Kip1}$ mRNA at 2 h instead of 4 h after treatment with forskolin, but the level of induction was 25-fold (see Fig. S4B in the supplemental material). These results suggest that Phox2a activation and onset of Phox2a-mediated transcription are influenced by the phosphorylation status of the entire phosphoserine cluster; on the other hand, complete dephosphorylation of this phosphoserine cluster, modeled by S202A/S206A/S208APhox2a, is required for subsequent Ser153 phosphorylation (Fig. 4), which terminates Phox2a activity. Thus, the phosphorylation status of the phosphoserine cluster could serve as a sensitive modulator of the amplitude and duration of Phox2a activation (see Discussion). Based on this interpretation, the triple-alanine mutation eliminates this modulatory function and accelerates both the onset of $p27^{Kip1}$ transcription and the subsequent inactivation of Phox2a, thus resulting in an early but modest induction of $p27^{Kip1}$ transcription.

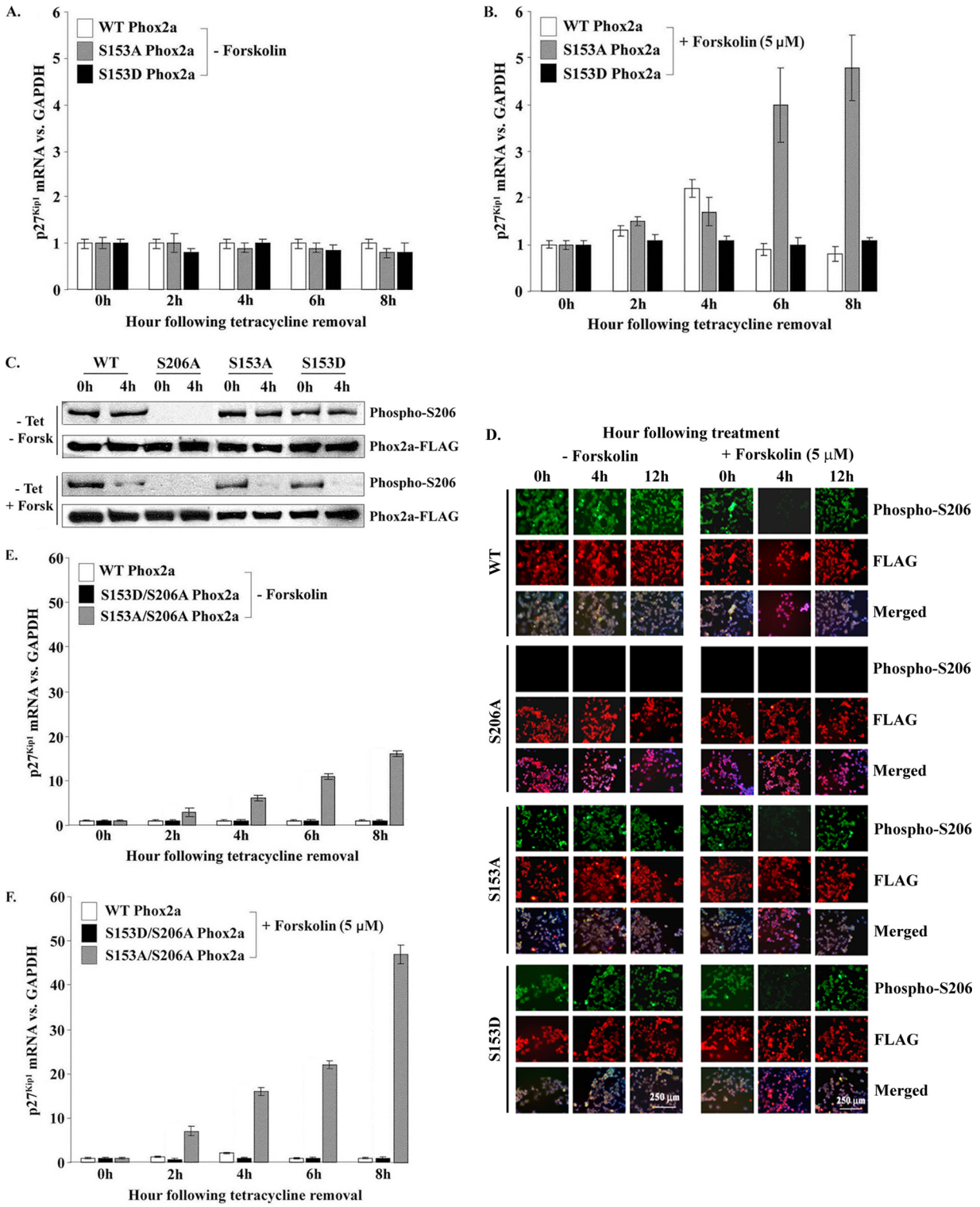

FIG. 4. Real-time PCR quantification of endogenous $p27^{Kip1}$ mRNA using total RNA isolated from CAD cell lines that express the indicated Phox2a proteins via the Tet-off system (see Fig. S2 in the supplemental material). (A and E) Without (−) forskolin; (B and F) with (+) forskolin added concurrently with tetracycline removal. Three independent RNA isolations were analyzed by PCR using identical triplicates. Quantification used GAPDH as an internal control. Fold induction is relative to 0 h. (C and D) Immunoblots (C) and immunofluorescence microscopy (D) using the phospho-S206 Phox2a antibody. Lysates in panel C or cultures in panel D were from the indicated CAD-Phox2a-FLAG expressing cell lines grown without (−) tetracycline for 72 h and treated for an additional 0 to 12 h with (+) or without (−) forskolin (5 μM). Error bars indicate standard deviations.

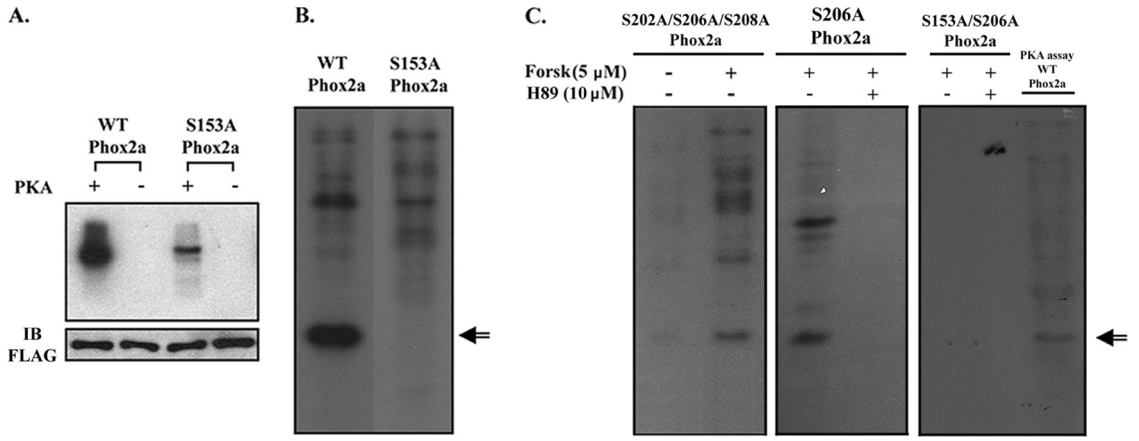

FIG. 5. (A) Purified WT Phox2a-FLAG and S153Phox2a proteins were incubated with (+) or without (−) the catalytic subunit of PKA and analyzed by 10% SDS-PAGE and autoradiography. A FLAG immunoblot (IB) of Phox2a proteins used in kinase reactions is shown. (B) Tryptic digests of in vitro PKA-phosphorylated WT Phox2a and S153APhox2a proteins, analyzed by 40% polyacrylamide alkaline gel electrophoresis and autoradiography. The arrow indicates the major phosphorylated peptide. (C) Tryptic digests of metabolically labeled S202A/S206A/S208APhox2a, Ser206APhox2a, and S153A/S206APhox2a proteins, analyzed by 40% polyacrylamide alkaline gel electrophoresis and autoradiography. Phox2a proteins were expressed by tetracycline removal for 72 h. Metabolic labeling with [^{32}P]orthophosphate was for 2.5 h for S202A/S206A/S208APhox2a, concurrent with (+) or without (−) addition of forskolin. Ser206APhox2a and S153A/S206APhox2a were incubated for 4.5 h with (+) forskolin, with or without concurrent addition of H89 as indicated; [^{32}P]orthophosphate was added for the last 3.5 h. Immunoprecipitated Phox2a proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes; the protein bands of interest were excised, digested with trypsin, electrophoresed on 40% alkaline polyacrylamide gels, and analyzed by autoradiography. WT Phox2a was in vitro phosphorylated by PKA and processed in parallel with the metabolically labeled S153A/S206APhox2a samples.

To test directly if PKA-mediated phosphorylation of Ser153 is responsible for the early and weak p27^{Kip1} induction observed with the triple-alanine Phox2a mutant, we quantified cAMP-induced p27^{Kip1} expression in S202A/S206A/S208APhox2a-expressing cells with and without addition of the PKA inhibitor H89 at 2 h. In the presence of H89, p27^{Kip1} transcription increased continuously for the duration of the time course (Fig. 6C), while OA treatment did not alter the expression profile of p27^{Kip1}. These results provide additional support for a direct role of PKA in terminating Phox2a activation after dephosphorylation of the phosphoserine cluster.

cAMP-dependent phosphorylation of Phox2a regulates its DNA binding capacity. ChIP assays have demonstrated that activation of the cAMP pathway regulates the binding of Phox2a to HD site 3 of the endogenous p27^{Kip1} promoter (29). Using the same method, we investigated the DNA binding potential of Phox2a-FLAG proteins containing mutations of Ser206 and Ser153, in CAD cells, as a function of treatment with forskolin for 4 h (Fig. 7A). S206APhox2a displayed a fivefold increase in binding to HD site 3, independent of forskolin treatment. In contrast, S206DPhox2a lacked binding to HD site 3, irrespective of treatment with forskolin. S153APhox2a resulted in eightfold-increased binding to the p27^{Kip1} promoter, but only with forskolin treatment, supporting that cAMP-dependent dephosphorylation of Ser206 is necessary for DNA binding. The double mutant S153A/S206APhox2a exhibited a greater increase in DNA binding with forskolin treatment than without (15-fold versus 8-fold). This is consistent with an additional contribution of Ser202 and Ser208 dephosphorylation to cAMP-induced Phox2a activation. Importantly, S153D/S206APhox2a failed to bind the endogenous p27^{Kip1} promoter, further demonstrating that phosphorylation of Ser153 is dominant to dephosphorylation of Ser206.

We confirmed the ChIP results by in vitro DNA binding assays, including electrophoretic mobility shift assays (data not shown), and Southwestern blot assays (Fig. 7B). Phox2a proteins (WT and the indicated mutants) were purified from cells grown for 4 h with forskolin treatment, with or without addition of OA. Following SDS-PAGE, purified proteins were transferred to nitrocellulose and incubated with ^{32}P-radiolabeled DNA containing WT or a mutant HD site 3. The results clearly show that WT Phox2a, purified from forskolin-stimulated cells, bound the HD site 3 of the p27^{Kip1} promoter, and this binding was inhibited when Phox2a was purified from cells treated with forskolin plus OA. In contrast, S206APhox2a bound HD site 3 irrespective of forskolin or OA treatment, and S206DPhox2a did not bind HD site 3 under any conditions. Likewise, S153A/S206APhox2a displayed constitutive binding to HD site 3, whereas S153DS206APhox2a failed to bind under any conditions.

Alanine substitutions of Phox2a Ser206 and Ser153 accelerate CAD cell neuronal differentiation. We investigated whether cAMP-mediated Phox2a modifications influence neuronal differentiation of our CAD cell lines. Neuronal differentiation, monitored by immunofluorescence detection of neurites, occurs within 12 h following expression of Phox2a-FLAG by tetracycline removal and concurrent treatment with forskolin (29) (Fig. 8A). In the absence of forskolin treatment, ectopic Phox2a-FLAG does not induce neuronal differentiation (Fig. 8B). All serine-to-alanine Phox2a mutants exhibited accelerated neurite formation in response to forskolin treatment (Fig. 8A), whereas S206DPhox2a and S153DPhox2a lacked

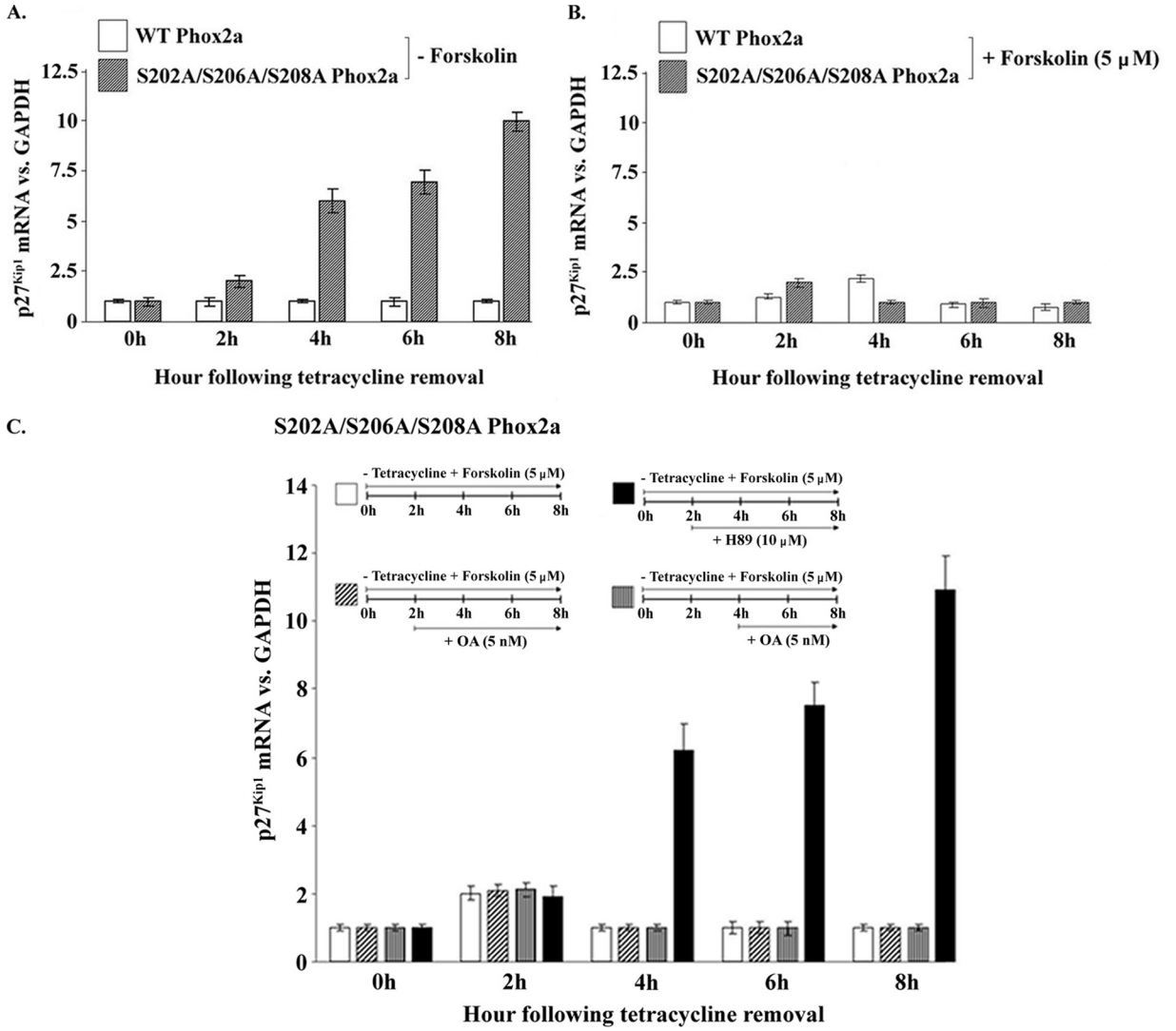

FIG. 6. Real-time PCR quantification of endogenous $p27^{Kip1}$ mRNA using total RNA isolated from CAD cell lines that express the indicated Phox2a proteins via the Tet-off system (see Fig. S2 in the supplemental material). (A) Without (−) forskolin; (B) with (+) forskolin added concurrently with tetracycline removal. (C) Forskolin was added at 0 h; H89 or OA was added 2 or 4 h after addition of forskolin. Three independent RNA isolations were analyzed by PCR using identical triplicates. Quantification used GAPDH as an internal control. Induction (*n*-fold) is relative to 0 h. Error bars indicate standard deviations.

neuronal differentiation (Fig. 8B; see Fig. S5 in the supplemental material).

Of special note is the accelerated appearance of the neuronal phenotype within 1 to 2 h after expression of S206APhox2a and S153A/S206APhox2a in the presence of forskolin. Cells expressing these Phox2a mutants exhibit robust $p27^{Kip1}$ mRNA induction (≈8-fold) at 2 h following activation of the cAMP pathway (Fig. 3C and 4F). To examine whether this accelerated neuronal phenotype is directly attributable to the early and enhanced expression of $p27^{Kip1}$, we depleted endogenous $p27^{Kip1}$ mRNA with siRNA. Indeed, the accelerated neuronal differentiation was abrogated upon transfection of $p27^{Kip1}$ siRNA (Fig. 8C; see Fig. S6 in the supplemental material). This result confirms the requirement of $p27^{Kip1}$ transcription for coupling cell cycle exit of neural progenitors with neuronal differentiation (29). Lastly, we found that treatment with OA inhibited cAMP-induced neuronal differentiation only in cells that express S153APhox2a, providing additional evidence that Ser206 dephosphorylation triggered by the cAMP pathway is required for $p27^{Kip1}$ transcription and neuronal differentiation (Fig. 8D).

DISCUSSION

cAMP-mediated dephosphorylation activates Phox2a. The cAMP pathway coordinates cell cycle exit of CAD neural progenitors with neuronal differentiation by activating Phox2a, which mediates transcription of $p27^{Kip1}$ (29). Here, we determined the mechanism by which the cAMP pathway activates Phox2a in the noradrenergic CAD cell line (see model in Fig. 9).

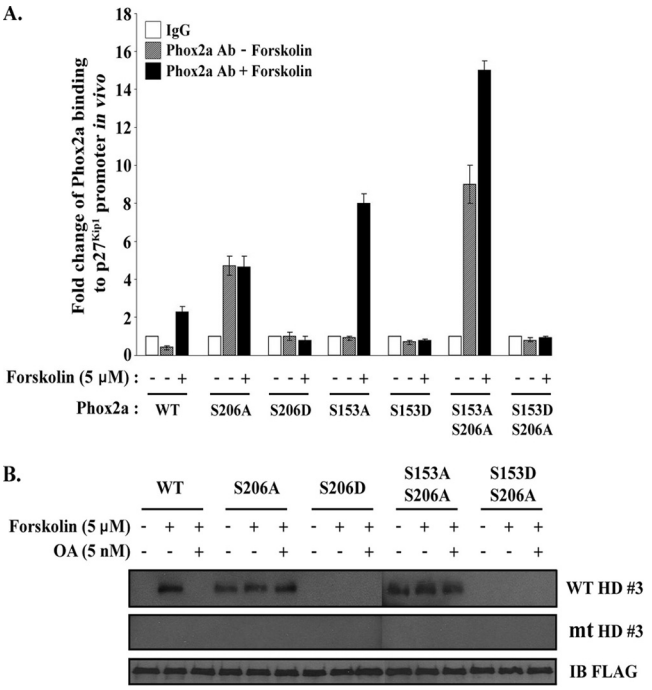

FIG. 7. (A) ChIP performed with CAD cell lines expressing WT and the indicated Phox2a mutant proteins. Ectopic Phox2a was expressed by tetracycline removal for 72 h (29) with (+) or without (−) forskolin for 4 h. PCR quantification of HD site 3 DNA of the $p27^{Kip1}$ promoter in immunoglobulin G (IgG) or Phox2a immunoprecipitates is shown. Data are expressed as changes of Phox2a binding to the $p27^{Kip1}$ promoter quantified relative to IgG. Results are from at least three independent experiments. Error bars indicate standard deviations. (B) Southwestern blot analyses of purified WT and mutant Phox2a proteins isolated from the respective CAD cell lines grown with (+) or without (−) forskolin and OA for 4 h, employing ^{32}P-radiolabeled WT and mutant (mt) HD probes. FLAG immunoblots (IB) of WT and mutant Phox2a-FLAG proteins were run in parallel.

(i) Inactive Phox2a. Our results are consistent with a model in which inactive Phox2a is phosphorylated in a cluster of proline-directed phosphoserine sites (Fig. 1), located at the C-terminal repression domain of Phox2a (38), until activation of the cAMP pathway. The cluster of phosphorylation sites identified by MS includes Ser202, Ser206, and Ser208, with Ser206 having the most prominent phosphorylation of Phox2a, and based on our mutational analysis, being the most important for regulating Phox2a activation (Fig. 3B).

(ii) Active Phox2a. Activation of the cAMP pathway mediates the critical dephosphorylation of Ser206 via an OA-sensitive step, demonstrated by immunodetection with phospho-S206-specific Phox2a antibody (Fig. 2A). Dephosphorylation of Ser206, modeled by S206APhox2a, allows binding of Phox2a to HD site 3 of the $p27^{Kip1}$ promoter (Fig. 7), resulting in transcription of the $p27^{Kip1}$ gene (Fig. 3). Importantly, inhibition of PKA by H89 or inhibition of the phosphatase by OA abrogates both dephosphorylation of Ser206 and neuronal differentiation (Fig. 2C), suggesting that cAMP-mediated dephosphorylation of Ser206 of Phox2a is functionally important. This cAMP-mediated dephosphorylation of Ser206 occurs in the noradrenergic CAD cell line as well as in primary cultures of neural crest cells stimulated with cAMP-elevating agents (data not shown). Significantly, the dephosphorylation of Ser206 coincides with the interval of $p27^{Kip1}$ transcription determined in our earlier study (29).

The kinase and phosphatase that act on the phosphoserine cluster remain to be determined. Inhibition of Phox2a activation by low concentrations (1 to 10 nM) of OA suggests that the phosphatase belongs to the protein phosphatase 2A family, as suggested in an earlier study (7). On the other hand, cyclin-dependent and mitogen-activated protein kinases are likely candidates for phosphorylation of Ser202, Ser206, and Ser208 since they are proline-directed phosphoserine sites.

Time-dependent inactivation of Phox2a. Activation of the cAMP pathway induces $p27^{Kip1}$ transcription in a defined interval prior to neuronal differentiation (29), after which $p27^{Kip1}$ transcription is turned off. S206APhox2a mediates constitutive $p27^{Kip1}$ transcription, but upon activation of the cAMP pathway the pattern resembles that of WT Phox2a; i.e., it displays peak transcription in a defined interval (4 h) and then returns to basal expression (Fig. 3). We provide evidence that a PKA-mediated phosphorylation of Ser153, located adjacent to the HD, is responsible for this time-dependent inactivation of Phox2a. PKA-mediated Ser153 phosphorylation, modeled by aspartic acid substitution at that site, prevents association of Phox2a with the $p27^{Kip1}$ promoter (Fig. 7), thereby terminating $p27^{Kip1}$ transcription (Fig. 4 and 5). Thus, the mechanism of Phox2a regulation involves two distinct cAMP signaling-dependent events separated by a defined time delay: (i) dephosphorylation of the C-terminal phosphoserine cluster facilitates Phox2a DNA binding and $p27^{Kip1}$ promoter occupancy (Fig. 7), and (ii) phosphorylation by PKA of Ser153 (Fig. 5) interferes with the binding of Phox2a to the $p27^{Kip1}$ promoter (Fig. 7), thereby terminating $p27^{Kip1}$ transcription.

We propose that the time delay that separates these two events is determined by the rate of complete dephosphorylation of the phosphoserine cluster. We base this proposal on functional studies which demonstrate that the PKA-mediated phosphorylation of Ser153 occurs only after the dephosphorylation of Ser202, Ser206, and Ser208, as modeled by the triple-alanine S202A/S206A/S208APhox2a mutant (Fig. 6). This Phox2a mutant displays constitutive, cAMP-independent expression of $p27^{Kip1}$ mRNA, indicating that the protein is transcriptionally functional. In contrast, the effect of the cAMP-mediated regulation on the triple-alanine Phox2a mutant is the inhibition of $p27^{Kip1}$ transcription after the 2-h interval. Importantly, inhibition of PKA by H89 after the 2-h interval reverses the premature termination of $p27^{Kip1}$ transcription mediated by S202A/S206A/S208APhox2a and allows constitutive $p27^{Kip1}$ expression similar to that for S153A/S206APhox2a. Treatment with OA has no effect on $p27^{Kip1}$ transcription, clearly distinguishing dephosphorylation of Ser202, Ser206, and Ser208 in response to activation of the cAMP pathway from the PKA-mediated phosphorylation of Ser153 (Fig. 6C).

Our interpretation of these results is that phosphorylation of the phosphoserine cluster prevents Ser153 phosphorylation. It is not until the phosphoserine cluster has been completely dephosphorylated that PKA can catalyze phosphorylation of Ser153 to terminate Phox2a transcriptional activity. Initial activation of Phox2a requires partial dephosphorylation of the phosphoserine cluster. Our results with the S206APhox2a mu-

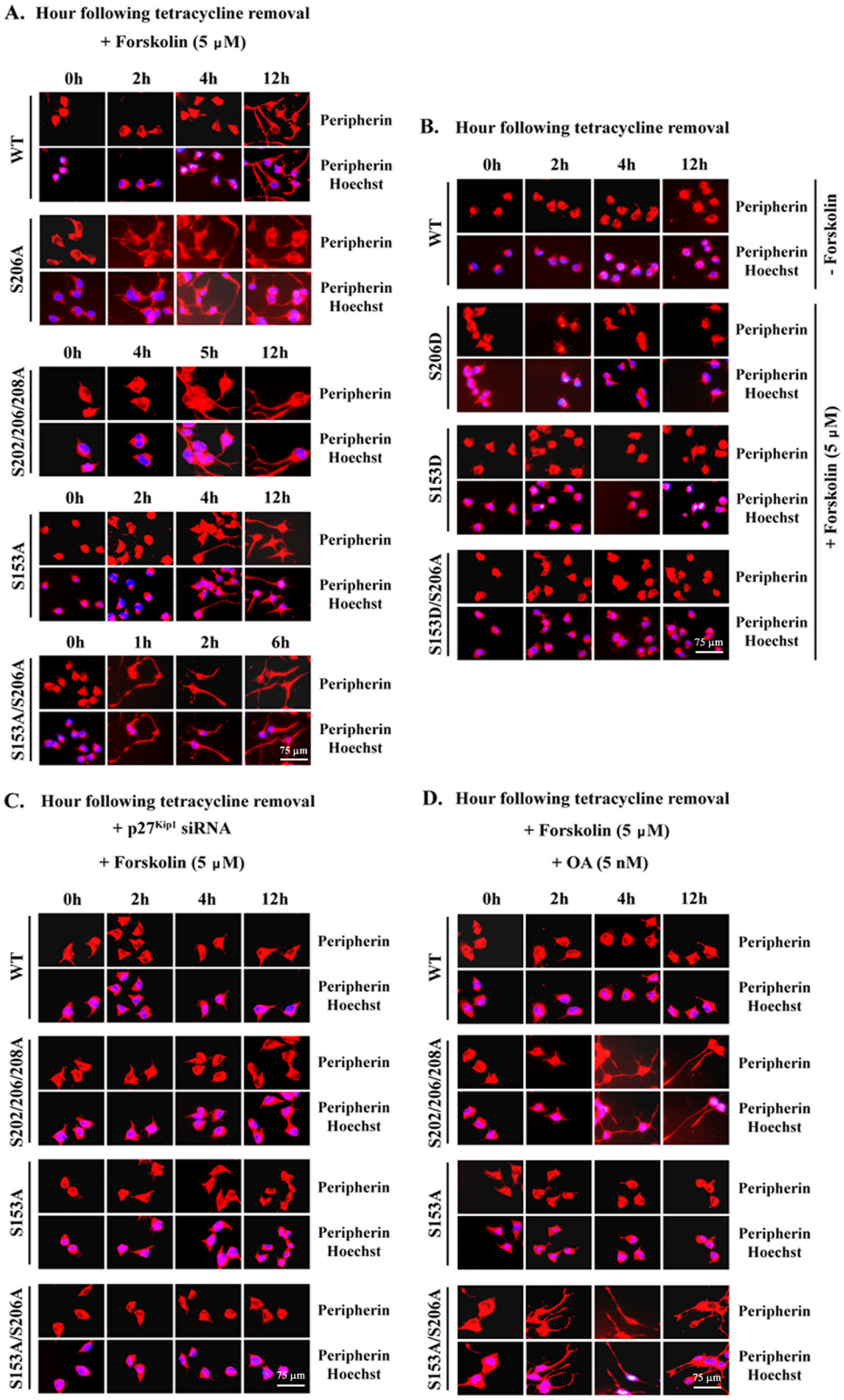

FIG. 8. Immunofluorescence microscopy of peripherin, using CAD cell lines expressing WT Phox2a-FLAG and the indicated Phox2a mutants by tetracycline removal. (A, B, and D) The indicated cell lines were grown following concurrent removal of tetracycline and addition of forskolin (A and B) and with cotreatment with OA (5 nM) (D). CAD-Phox2a-FLAG mutant cell lines grown without treatment with forskolin are shown in Fig. S5 in the supplemental material. (C) Knockdown of $p27^{Kip1}$ mRNA by transfection of siRNA (100 nM) in the indicated cell lines, performed as previously described (29). siRNA controls are shown in Fig. S6 in the supplemental material. Merged images show immunostaining with the peripherin antibody (red) and DNA staining with Hoechst stain (blue).

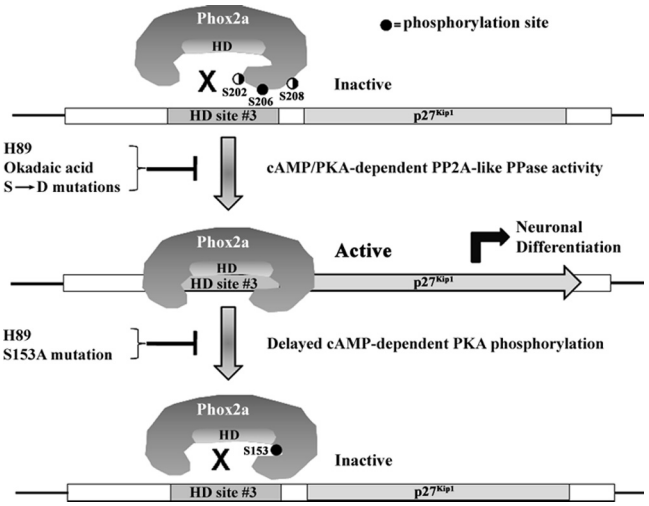

FIG. 9. Mechanism of the time-dependent activation of Phox2a by cAMP signaling. Phosphorylation of Ser206 (the major phosphorylation site, depicted by filled circle), Ser202, and Ser208 indicates the inactive state of Phox2a. Activation of the cAMP pathway mediates the dephosphorylation of Ser206 via an OA-sensitive phosphatase. Active Phox2a binds to HD site 3 of the $p27^{Kip1}$ promoter and induces $p27^{Kip1}$ transcription. Upon complete dephosphorylation of Ser202, Ser206, and Ser208, Phox2a-mediated transcription is terminated via a PKA-mediated phosphorylation of Phox2a Ser153. This represents a novel mechanism of transcription factor activation by the cAMP pathway. The same signal determines the onset and duration of Phox2a-mediated transcription with a built-in time delay.

tant are consistent with this interpretation. Specifically, S206APhox2a is active in the absence of cAMP signaling (Fig. 3B). Additional time is required for further dephosphorylation of Ser202 and Ser208, and possibly even Ser183/Thr185, which was identified as a minor proline-directed phosphorylation site. As a result, a defined delay between the initial activation of Phox2a and the subsequent Ser153-dependent inactivation is established. We conclude that PKA mediates the delayed termination of $p27^{Kip1}$ transcription by phosphorylating Ser153 and that this event is responsive to the phosphorylation status of the C-terminal phosphoserine cluster. Thus, Phox2a is intrinsically programmed to be active for a defined period of time. This mechanism of Phox2a regulation by the cAMP pathway contrasts with the classic cAMP-mediated stimulus-response mechanism of CREB activation (11, 12).

Role of multisite phosphorylation in Phox2a regulation. Multisite phosphorylation of phosphoserine clusters has been observed and characterized in other transcription factors (11, 31). In the case of inhibitory multisite phosphorylations of transcription factors, the intensity of the activating signal determines the degree of dephosphorylation, which in turn determines the magnitude and fine-tuning of transcriptional activation. Interestingly, the mechanism of Phox2a activation by cAMP signaling offers the following novel regulation: the rate of dephosphorylation of the phosphoserine cluster of Phox2a acts to establish a time delay between two regulatory events, with the first event activating and the second inactivating Phox2a, both in response to the same initial stimulus. Our data suggest that the phosphoserine cluster of Phox2a undergoes a time-dependent, graded dephosphorylation, raising the question of the mechanism modulating this process.

Reversible phosphorylation of Ser/Thr-Pro is a signaling mechanism known to regulate transcription (38, 40, 41) and cell cycle progression (27, 34, 43). An aspect of this regulation involves the *cis*/*trans* isomerization of prolyl residues catalyzed by prolyl isomerase Pin1, which recognizes only phosphorylated Ser/Thr-Pro bonds (26). It is well established that some kinases and phosphatases, including protein phosphatase 2A, require the *trans* isomer of Ser/Thr-Pro sequences (39). Thus, it is interesting to speculate that regulated prolyl isomerization of Phox2a by Pin1 could be involved in regulating Phox2a activity. Interestingly, recent studies have shown that Pin1 regulates $p27^{Kip1}$ transcription by inhibiting the transcriptional activity of the tumor suppressor FOXO (6). Therefore, one possibility is that prolyl isomerization of Phox2a facilitates dephosphorylation of the Ser-Pro cluster, promoting activation of Phox2a; alternatively, prolyl isomerization of Phox2a may maintain the inactive state by facilitating phosphorylation of the Ser-Pro cluster. Thus, a dynamic balance of opposing phosphatase and kinase activities may contribute to the overall rate of dephosphorylation of the Ser-Pro cluster. The mechanistic nature of the delay between the activating dephosphorylation of Phox2a and the inactivating phosphorylation of Ser153 is currently under study.

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REFERENCES

1. **Adachi, M., and E. Lewis.** 2002. The paired-like homeodomain protein, arix, mediates protein kinase A-stimulated dopamine β-hydroxylase gene transcription through its phosphorylation status. J. Biol. Chem. **277:**22915–22924.
2. **Anderson, D. J., J. F. Carnahan, A. Michelsohn, and P. H. Patterson.** 1991. Antibody markers identify a common progenitor to sympathetic neurons and chromaffin cells *in vivo* and reveal the timing of commitment to neuronal differentiation in the sympathoadrenal lineage. J. Neurosci. **11:**3507–3519.
3. **Benjanirut, C., M. Paris, W.-H. Wang, S. Hong, K. Kim, R. L. Hullinger, and O. M. Andrisani.** 2006. The cAMP pathway via CREB, in synergy with BMP2, regulates transcription from the mammalian Phox2a promoter via cAMP-response-element binding sites. J. Biol. Chem. **281:**2969–2981.
4. **Bilodeau, M. L., T. Boulineau, R. L. Hullinger, and O. M. Andrisani.** 2000. Cyclic AMP signaling functions as a bimodal switch in sympathoadrenal cell development in cultured primary neural crest cells. Mol. Cell. Biol. **20:**3004–3014.
5. **Bilodeau, M., M. Ji, M. Paris, and O. M. Andrisani.** 2005. Adenosine signaling regulates differentiation of both neural crest-derived and CNS-derived catecholaminergic neurons. Mol. Cell. Neurosci. **29:**394–404.
6. **Brenkman, A. B., P. L. J. de Keizer, N. J. F. van der Broek, P. van der Groep, P. J. van Diest, A. van der Horst, A. M. M. Smits, and M. T. Burgering.** 2008. The peptidyl-isomerase Pin1 regulates $p27^{Kip1}$ expression through inhibition of forkhead BoxO tumor suppressor. Cancer Res. **68:**7597–7605.
7. **Chen, S., M. Ji, M. Paris, R. L. Hullinger, and O. M. Andrisani.** 2005. The cAMP pathway regulates both transcription and activity of the paired homeobox transcription factor Phox2a required for development of neural crest-derived and CNS-derived catecholaminergic neurons. J. Biol. Chem. **280:**41025–41036.
8. **Cowley, D. O., and B. J. Graves.** 2000. Phosphorylation represses Eta-1 DNA-binding by reinforcing autoinhibition. Genes Dev. **14:**366–376.
9. **Cremisi, F., A. Philpott, and S. Ohnuma.** 2003. Cell cycle and cell fate interactions in neural development. Curr. Opin. Neurobiol. **13:**26–33.
10. **Dadd, C. A., R. G. Cook, and C. D. Allis.** 1993. Fractionation of small tryptic phosphopeptides by alkaline PAGE followed by amino acid sequencing. BioTechniques **14:**266–273.
11. **Gardner, K. H., and M. Montminy.** 2005. Can you hear me now? Regulating transcriptional activators by phosphorylation. Sci. STKE. **301:**pe44.

12. **Gonzalez, G. A., and M. R. Montminy.** 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell **59:**675–680.
13. **Guo, S., S. W. Wilson, S. Cooke, A. B. Chitnis, W. Driever, and A. Rosenthal.** 1999. Mutations in the zebrafish unmask shared regulatory pathways controlling the development of catecholaminergic neurons. Dev. Biol. **208:**473–487.
14. **Hall, M. C., D. E. Jeong, J. T. Henderson, E. Choi, S. C. Bremmer, A. B. Iliuk, and H. Charbonneau.** 2008. Cdc28 and Cdc14 control stability of the anaphase-promoting complex inhibitor Acm1. J. Biol. Chem. **283:**10396–10407.
15. **Handen, J. S., and H. F. Rosenberg.** 1997. An improved method for southwestern blotting. Front. Biosci. **2:**C9–C11.
16. **Hirsch, M. R., M. C. Tiveron, F. Guillemot, J. F. Brunet, and C. Goridis.** 1998. Control of noradrenergic differentiation and Phox2a expression MASH1 in the central and peripheral nervous system. Development **125:**599–608.
17. **Holmberg, C. I., S. E. F. Tran, J. E. Eriksson, and L. Sistonen.** 2002. Multisite phosphorylation provides sophisticated regulation of transcription factors. Trends Biochem. Sci. **27:**619–627.
18. **Howard, M. J.** 2005. Mechanisms and perspectives on differentiation of autonomic neurons. Dev. Biol. **277:**271–286.
19. **Iulianella, A., M. Sharma, M. Durnin, G. B. Vanden Heuvel, and P. A. Trainor.** 2008. *Cux2* (*Cut12*) integrates neural progenitor development with cell-cycle progression during spinal cord neurogenesis. Development **135:**729–741.
20. **Keshvara, L. M., C. C. Isaacson, T. M. Yankee, R. Sarac, M. L. Harrison, and R. L. Geahlen.** 1998. Syk- and Lyn-dependent phosphorylation of Syk on multiple tyrosines following B cell activation includes a site that negatively regulates signaling. J. Immunol. **161:**5276–5283.
21. **Ko, L. J., and C. Prives.** 1996. p53: puzzle and paradigm. Genes Dev. **10:**1054–1072.
22. **Le Douarin, N. M., S. Creuzet, G. Couly, and E. Dupin.** 2004. Neural crest plasticity and its limits. Development **131:**4637–4650.
23. **Lee, Y. S., F. Liu, and N. Segil.** 2006. A morphogenetic wave of *p27^{Kip1}* transcription directs cells cycle exit during organ of Corti development. Development **133:**2817–2826.
24. **Lo, L., X. Morin, J. F. Brunet, and D. J. Anderson.** 1999. Specification of neurotransmitter identity by Phox2 proteins in neural crest stem cells. Neuron **22:**693–705.
25. **Lo, L., M. C. Tiveron, and D. J. Anderson.** 1998. MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. Development **125:**609–620.
26. **Lu, K. P., G. Finn, T. H. Lee, and L. K. Nicholson.** 2007. Prolyl cis-trans isomerization as a molecular timer. Nat. Chem. Biol. **3:**619–629.
27. **Lu, K. P., and X. Z. Zhou.** 2007. The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signaling and disease. Nat. Rev. Mol. Cell Biol. **8:**904–916.
28. **Morin, X., H. Cremer, R. Hirsch, R. P. Kapur, C. Goridis, and J. F. Brunet.** 1997. Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene Phox2a. Neuron **18:**411–423.
29. **Paris, M., W.-H. Wang, M.-H. Shin, D. S. Franklin, and O. M. Andrisani.** 2006. The transcription factor Phox2a coordinates cell cycle exit and catecholaminergic neuron differentiation in central nervous system-derived and neural crest progenitors via transcriptional induction of p27^{Kip1}. Mol. Cell. Biol. **26:**8826–8839.
30. **Qin, B. Y., C. Liu, S. S. Lam, H. Srinath, R. Delston, J. J. Correia, R. Derynck, and K. Lin.** 2003. Crystal structure of IFR-3 reveals mechanism of autoinhibition and virus-induced phosphoactivation. Nat. Struct. Biol. **10:**913–921.
31. **Rao, A., C. Luo, and P. G. Hogan.** 1997. Transcription factors of the NFAT family: regulation and function. Annu. Rev. Immunol. **15:**707–747.
32. **Reissmann, E., U. Ernsburger, P. H. Francis-West, D. Rueger, P. D. Brickell, and H. Rohrer.** 1996. Involvement of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons. Development **122:**2079–2088.
33. **Schneider, C., H. Wicht, J. Enderich, M. Wegner, and H. Rohrer.** 1999. Bone morphogenetic proteins are required *in vivo* for the generation of sympathetic neurons. Neuron **24:**861–879.
34. **Shen, M., P. T. Stukenberg, P. W. Kirschner, and K. P. Lu.** 1998. The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins. Genes Dev. **12:**706–720.
35. **Stanke, M., D. Junghans, M. Geissen, C. Goridis, U. Ernsberger, and H. Rohrer.** 1999. The Phox2 homeodomain proteins are sufficient to promote the development of sympathetic neurons. Development **126:**4087–4094.
36. **Suri, C., B. P. Fung, A. S. Tischler, and D. M. Chikaraishi.** 1993. Catecholaminergic cell lines from the brain and adrenal glands of tyrosine hydroxylase-SV40 T antigen transgenic mice. J. Neurosci. **13:**1280–1291.
37. **Swanson, D. J., E. Zellmer, and E. J. Lewis.** 1997. The homeodomain protein Arix interacts synergistically with cyclic AMP to regulate expression of neurotransmitter biosynthetic genes. J. Biol. Chem. **272:**27382–27392.
38. **Swanson, D. J., M. Adachi, and E. J. Lewis.** 2000. The homeodomain protein Arix promotes protein kinase A-dependent activation of the dopamine β-hydroxylase promoter through multiple elements and interaction with the coactivator cAMP-response element-binding protein-binding protein. J. Biol. Chem. **275:**2911–2923.
39. **Wulf, G., G. Finn, F. Suizu, and K. P. Lu.** 2005. Phosphorylation-specific prolyl isomerization: is there an underlying theme? Nat. Cell Biol. **7:**435–441.
40. **Xu, Y. X., Y. Hirose, X. Z. Zhou, K. P. Lu, and J. L. Manley.** 2003. Pin1 modulates the structure and function of human RNA polymerase II. Genes Dev. **17:**2765–2776.
41. **Xu, Y. X., and J. L. Manley.** 2007. Pin1 modulates RNA polymerase II activity during the transcription cycle. Genes Dev. **21:**2950–2962.
42. **Zellmer, E., Z. Zhang, D. Greco, J. Rhodes, S. Cassel, and E. J. Lewis.** 1995. A homeodomain protein selectively expressed in noradrenergic tissue regulates transcription of neurotransmitter biosynthetic genes. J. Neurosci. **15:**8109–8120.
43. **Zhou, X. Z., O. Kops, A. Werner, P. J. Lu, M. Shen, G. Stoller, G. Külleertz, M. Stark, G. Fischer, and K. P. Lu.** 2000. Pin1-dependent prolyl isomerization regulates dephosphorylation of Cdc25C and tau proteins. Mol. Cell **6:**873–883.